FORM PTO-1390 (REV 12-2-99) U.S. DEPARTMENT OF COMMERCE PATTENT AND TRADEMARK OFFICE

JC11 Rec'd PCT/PTO SEP 2 6 2001

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER: 031676.0208

U S APPLICATION NO (if known, see 37 CFR 1 5): 09/937477 To Be Assigned

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/US00/07989	March 27, 2000	March 26, 1999

TITLE OF INVENTION

SPECIFIC BINDING ASSAY FOR DOCOSAHEXAENOIC ACID

APPLICANT(S) FOR DO/EO/US

F.C. Thomas ALLNUTT, et al.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ has been transmitted by the International Bureau.
   c. ☒ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ have been transmitted by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☐ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included
13. ☐ A **FIRST** preliminary amendment.
    ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☒ Other items or information:

Unexecuted Declaration and Power of Attorney of Patent Application

JC03 Rec'd PCT/PTO 26 SEP 2001

U.S. APPLICATION NO (IF KNOWN, SEE 37 CFR 1.7)	INTERNATIONAL APPLICATION NO	ATTORNEY'S DOCKET NUMBER
To Be Assigned 09/937477	PCT/US00/07989	031676.0208

17. ☒ The following fees are submitted:

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1)-(5)):

	CALCULATIONS PTO USE ONLY
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2) paid to USPTO and International Search Report not prepared by the EPO or JPO **$1000.00**	
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO..... **$860.00**	
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO **$710.00**	
International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4)..... **$690.00**	**$690.00**
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) **$100.00**	

ENTER APPROPRIATE BASIC FEE AMOUNT =					**$690.00**	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).					**$**	
CLAIMS	NUMBER FILED	NUMBER EXTRA		RATE		
Total claims	-20 =		x	$18.00	**$**	
Independent claims	-3 =		x	$80.00	**$**	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+	$270.00	**$**	
TOTAL OF ABOVE CALCULATIONS =					**$690.00**	
Reduction of ½ for filing by small entity, if applicable. A small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28).					**$ 0.00**	
SUBTOTAL =					**$690.00**	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f)). +					**$**	
TOTAL NATIONAL FEE =					**$690.00**	
Fee for recording the enclose assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +					**$**	
TOTAL FEES ENCLOSED =					**$690.00**	
					Amount to be refunded:	**$**
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a. ☐ A check in the amount of $ _____ to cover the above fees is enclosed.

b. ☒ Please charge my Deposit Account No. 50-1640 in the amount of **$690.00** to cover the above fees.
A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any fees which may be required, or credit any overpayment
To Deposit Account No. 50-1640 A duplicate coy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

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NAME

34,698
REGISTRATION NUMBER

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS (37 CFR 1.9(f) AND 1.27(c)) - SMALL BUSINESS CONCERN			Docket No. 031676.0208
Application No. 09/937,477	Filing Date September 26, 2001	Patent No.	Issue Date

Applicant/ Patentee: **F.C. Thomas ALLNUTT *et al.***

Invention: **SPECIFIC BINDING ASSAY FOR DOCOSAHEXAENOIC ACID**

I hereby declare that I am:

☐ the owner of the small business concern identified below:

☐ an official of the small business concern empowered to act on behalf of the concern identified below:

NAME OF CONCERN: **Martek Biosciences Corp.**

ADDRESS OF CONCERN: **6480 Dobbin Road, Columbia, Maryland 21045, USA**

I hereby declare that the above-identified small business concern qualifies as a small business concern as defined in 13 CFR 121.3-18, and reproduced in 37 CFR 1.9(d), for purposes of paying reduced fees under Sections 41(a) and (b) of Title 35, United States Code, in that the number of employees of the concern, including those of its affiliates, does not exceed 500 persons. For purposes of this statement, (1) the number of employees of the business concern is the average over the previous fiscal year of the concern of the persons employed on a full-time, part-time or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when either, directly or indirectly, one concern controls or has the power to control the other, or a third party or parties controls or has the power to control both.

I hereby declare that rights under contract or law have been conveyed to and remain with the small business concern identified above with regard to the above-identified invention described in:

☐ the specification filed herewith with title as listed above.

☒ the application identified above.

☐ the patent identified above.

If the rights held by the above-identified small business concern are not exclusive, each individual, concern or organization having rights to the invention is listed below and no rights to the invention are held by any person, other than the inventor, who could not qualify as an independent inventor under 37 CFR 1.9(c) or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

Each person, concern or organization to which I have assigned, granted, conveyed or licensed or am under an obligation under contract or law to assign, grant, convey or license any rights in the invention is listed below:

☒ No such person, concern or organization exists.

☐ Each such person, concern or organization is listed below.

FULL NAME _____

ADDRESS _____

☐ Individual ☐ Small Business Concern ☐ Nonprofit Organization

FULL NAME _____

ADDRESS _____

☐ Individual ☐ Small Business Concern ☐ Nonprofit Organization

FULL NAME _____

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FULL NAME _____

ADDRESS _____

☐ Individual ☐ Small Business Concern ☐ Nonprofit Organization

Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING: George P. Barker

TITLE OF PERSON SIGNING OTHER THAN OWNER: Senior Vice President and General Counsel

ADDRESS OF PERSON SIGNING: Martek Biosciences Corporation
6480 Dobbin Road
Columbia, MD 21045

SIGNATURE: George P. Barker DATE: 1/16/02

Specific Binding Assay for Docosahexaenoic Acid

BACKGROUND

Field of the Invention

This invention relates to the detection and/or quantification of docosahexaenoic acid (DHA) in the presence of other fatty acids via a binding and detection assay. The invention also relates to the detection or quantification of DHA in biological samples such as blood, body fluids, or body tissues of individuals; in foods; or in microbial or algal lysates or other DHA production systems.

Review of the Related Art

The human brain and other neural tissues are highly enriched in long chain polyunsaturated fatty acids (PUFAs) which are thought to play an important role in modulating the structure, fluidity and function of the cell membranes of these tissues. DHA (4,7,10,13,16,19-docosahexaenoic acid; 22:6ω-3) is the most abundant of the fatty acids found in the structural components of grey matter of the human brain and other neural tissues. DHA cannot be synthesized *de novo* in humans, but there is some evidence that this ω-3 fatty acid can be synthesized by some cell types, such as astrocytes, if the appropriate long chain polyunsaturated fatty acids are provided in the diet (S. Moore, et al., 1991, *J. Neurochem.*, **56:**518-524). Most of the DHA found in the brain and retina cell membranes is believed to be obtained from dietary sources (see WO 94/28913).

The importance of providing polyunsaturated fatty acids during a period of rapid brain development to prevent irreparable damage to brain cells is well known in the art. Human infants appear to have a particularly poor ability to synthesize DHA, but deficiencies may be compensated for by feeding infants human breast milk, which is a rich source of essential fatty acids, particularly DHA and ARA (Sanders, et al, 1978, *J. Clin. Nutr.*, **31:**805-813). Recent studies comparing the performance on standard intelligence tests of children who were fed breast milk as babies to children who were fed commercial infant formulas as babies have suggested a dose response relationship between the proportion of mother's milk in the diet and subsequent IQ (Lucas, et al., 1992, *Lancet*, **339:**261-264). These studies

suggest that dietary intervention therapy can effect the levels of DHA available for structural development of the nervous system (see WO 96/40106).

Peroxisomal disorders are a group of degenerative neurological disorders characterized by increased levels of very long chain fatty acids, resulting from an impaired capacity of the effected individuals for degrading these fatty acids. These disorders are related in that they all appear to result from some defect localized in the subcellular organelles known as peroxisomes (Gordon, N., 1987, *Brain Development*, **9**:571-575). Recent studies of the polyunsaturated fatty acid composition of tissues in patients with peroxisomal disorders have shown that, even though the total amount of fatty acids in these tissues was normal, there are significant changes in the fatty acid composition of the patient's tissues. These patients have a significant decrease in the total amount of DHA and ARA in their serum lipid compositions. Serum plasmalogen levels are also depressed (see WO 96/40106).

Usher's syndrome is an autosomal recessive genetic disorder which is associated with the degeneration of visual cells, causing retinitis pigmentosa. The visual cells contain extremely large quantities of DHA esterified in the phospholipids of the photoreceptor membranes which make up the outer segments of the visual cells. Bazan and coworkers recently have found that the plasma phospholipids of Usher's patients contain significantly less DHA and ARA than the plasma phospholipids of unaffected individuals (Bazan, et al., 1986, *Biochem. Biophys. Res. Comm.*, **141**:600-604) (see WO 96/40106).

Reduction of DHA levels in brain tissue has been reported in patients with neurological disorders such as Alzheimer's disease, Attention Deficit Disorder, and negative symptom schizophrenia (Soderberg, et al., 1991, *Lipids*, **26**:421-425; Stevens, et al., 1995, *Am. J. Clin. Nutr.* **62**:671-8; Glen, et al., 1994, *Schizophrenia Res.* **12**:53-61). In addition researchers have found that patients suffering from other clinical conditions, such as senile dementia, diabetes-induced neuropathy, multiple sclerosis, and neuropathies associated with high doses of heavy metals such as lead, aluminum, and mercury also frequently have levels of DHA and/or ARA in their serum lipids which are significantly depressed in comparison to the levels found in healthy persons. For example, recent studies have established a correlation between

alternations in the levels of esterification of ARA into the phospholipids of platelets and the presence of schizoaffective disorders in patients (Demisch, et al., 1992, *Prostaglandins Leukot. Essent. Fatty Acids*, **46:**47-52) (see WO 94/28913).

Although researchers have made some progress in understanding neurodegenerative disorders such as Alzheimer's disease and various peroxisomal disorders, effective means of treating these disorders have remained elusive. Likewise, there has been a lack of progress in the development of effective therapeutic drugs to treat schizophrenia and other neurological disorders disclosed above (see WO 94/28913). Significantly, treatment with DHA has been shown to improve mental and physiological functioning. Martinez has repeatedly shown that the normalization of the DHA levels of children with peroxisomal disorders resulted in significant improvements of their physiological status (*e.g.*, improvements in visual function, muscle tone, awareness, and speech) and prognosis (Martinez, 1995, *J. Inher. Metab. Dis.* 18 Suppl. **1:**61-75).

Peroxisomal patients whose conditions had been dramatically deteriorating showed rapid improvements (*e.g.*, in as little as a few weeks) after DHA supplementation began (Martinez, 1995; Martinez, 1992, *New Dev. Fatty Acid Oxidation*, pp. 389-397, Wiley-Liss, Inc.). Further, patients' conditions continued to improve as DHA supplementation was continued or increased (Martinez, 1995; Martinez, 1992). These observations support a casual relationship between DHA supplementation and improvements in physiological status, improvements in prognosis, and/or decreases in diagnostic fatty acid ratios.

Alzheimer's patients have subnormal levels of brain DHA, and there have been recent reports on the supplementation of such patients with DHA which indicate that such supplementation leads to significant improvements in memory, communication (co-operation and speech), and psychological symptoms (delirium and orientation) (Miyanaga, et al., 1996, *Neurobiol. Aging* **17:**S73). The same study demonstrated similar improvements in cerebrovascular dementia patients who were given DHA supplementation.

In studies by Laugharne and Peet et al., supplementation of the diets of schizophrenic patients with omega-3 fatty acids improved the patients' outcome, particularly with regard to negative symptoms (Laugharne, et al., 1996, *Lipids* **31:**S-

163-165; Peet, et al., 1996, *Prostaglandins, Leukotri. and Essent. Fatty Acids*, **55:**71-75). For example, Peet and co-workers have observed that schizophrenic patients given DHA- and EPA-containing fish oil exhibited significant improvements in both schizophrenic symptoms and tardive dyskinesia over a 6 week period. Further, the improvement in schizophrenic symptoms was significantly correlated with increased levels of omega-3 fatty acids in erythrocytes (Peet, et al., 1996).

Hence, there is strong evidence that DHA deficiencies are associated with neurological disorders, and that administration of DHA can improve the condition of patients having these disorders. It is possible to obtain DHA via the consumption of cold water fish or fish oil. However, fish oil often has an unpleasant taste and may contain contaminants from environmental pollutants found in the oceans (Kyle, et al., U.S. Patent 5,407,957). Furthermore, fish oil contains eicosapentaenoic acid (hereinafter EPA), the consumption of which may have undesirable consequences, such as "blood thinning," or increased bleeding times, due to decreased platelet aggregation (Wojenski, et al., 1991, *Biochim. Biophys. Acta*, **1081:**33-38).

The prior art clearly demonstrates metabolic function of DHA and other LC-PUFAs. The body has developed systems that allow precise deposition of these LC-PUFAs. One component of the lipid utilization system is a class of specific binding proteins that recognize fatty acids, the fatty acid binding proteins (FABPs) (Glatz, et al., 1996, *Prog. Lipid Res.*, **35:**243-282). These are found in many tissues (heart, eye, liver and muscle), including the brain (Feng, et al., 1994, *Neuron.* **12:**895-908; Xu, et al., 1996, *J. Biol. Chem.*, **271:**24711-24714).

Brain Lipid Binding Protein (hereinafter BLBP) is a member of the FABP family of proteins. BLBP is expressed in brain, and studies have shown that the timing and spatial location of BLBP expression correlates with neuronal migration and differentiation (Feng, 1994; Kurtz, et al., 1994, *Development*, **120:**2637-2649). Glial growth factor mediates and is crucial for the proper expression of BLBP, which is essential for the establishment and maintenance of radial glial fiber systems during neuronal migration during brain development (Anton, et al., 1997, *Development*, **124:**3501-3510). Treating primary cerebellar cell cultures with anti-BLBP antibody prevented glial fascicle extension and could be reversed by either

washing out or pre-binding of BLBP to the antibody prior to addition of the anti-BLBP antibody to the cell culture (Anton, et al., 1997, *Development*, **124**:3501-3510). The body of knowledge for BLBP is still not sufficient to totally elucidate the importance of BLBP to brain development, but circumstantial evidence very strongly implicates BLBP involvement in some of the most important development activities for the brain.

Unlike other members of the FABP family, BLBP binds DHA with high affinity and specificity (Xu, 1996). According to Xu et al., BLBP binds DHA with a dissociation constant (Kd) of approximately 10 nM. Furthermore, competition assays demonstrated that DHA is a much more effective ligand for the BLBP that other fatty acids such as oleic acid (Xu, 1996). Taken together, the evidence that BLBP plays a role in neuronal differentiation and that BLBP binds DHA with high affinity support the correlation between brain levels of DHA and improved neural functioning.

Despite the evident physiological importance of DHA and other LC-PUFAs, measurement of these substances is currently done using cumbersome methods based on chromatographic separation of organically extracted and often chemically modified fatty acids (Christie, W.W., *Lipid Analysis*, 2nd Edition, 1982, Pergamon Press, NY; Stansby, Maurice E., *Fish Oils in Nutrition*, 1990, Van Nostrand Reinhold, NY). In view of the link between these fatty acids and important metabolic and disease states, there is a need for rapid assessment of the DHA status of patients, and of food or production systems. No such assay currently exits. A specific binding assay for DHA would provide the tools to quantitatively determine DHA in a sample and provide necessary knowledge to medicine, agriculture and industrial uses.

SUMMARY OF THE INVENTION

Accordingly, it is an object of this invention to overcome the problems and disadvantages associated with current strategies and designs for detecting and quantifying DHA.

It is a further object of this invention to provide binding and detection assays to detect and quantify DHA in biological samples such as blood, body fluids, or body tissues of individuals; in foods; or in microbial or algal lysates or other DHA

production systems. It is a related object of this invention to provide specific assay conditions which accelerate or increase the specificity of the binding of DHA by a protein specific for it, or which accelerate or increase the specificity of the detection of the DHA-protein complex. Another related object of the invention is to provide proteins or nucleic acid aptamers which specifically bind the DHA-protein complex.

It is another object of this invention to provide methods for using the DHA detection and quanitification system of this invention to detect or quantify DHA levels in biological samples such as blood, body fluids, or body tissues of individuals; in foods; or in microbial or algal lysates or other DHA production systems. These and other objects are met by one or more of the following embodiments.

In one embodiment, this invention provides a method for detecting the presence or amount of DHA in the presence of other fatty acids, by binding the DHA by a protein with differential specificity for DHA over other fatty acids and detecting the complex by any method which will distinguish between bound and unbound fatty acid. In a preferred embodiment, the protein used has an affinity for DHA that is at least half an order of magnitude greater than its affinity for other fatty acids. In another preferred embodiment, the DHA-protein complex is detected through binding by a protein or DNA aptamer specific for the complex.

Due to the desirability of treatment with or consumption of DHA and the problems associated with obtaining DHA from fish, methods have been developed to produce DHA, particularly DHA which is substantially free of EPA or other fatty acids, via cultivation of marine microorganisms. (WO 96/40106; U.S. Patents 5,407,957 and 5,397,591 to Kyle). DHA can also be obtained from other sources, which include but are not limited to animal tissue, eggs, and fish oil. The assays of this invention are useful for measuring the DHA content of these sources, and for monitoring the progress of isolation or production of DHA from these sources.

In the production of DHA by microorganisms, the time of optimal DHA synthesis in a production culture is subject to variation. In the absence of methods for real-time determination of DHA levels in the culture, it may be necessary to estimate the time of harvest, based on past data. (U.S. Patents 5,407,957 and 5,397,591 to Kyle). Using current methods of DHA detection, such as gas

chromatography. can lead to inefficiencies in DHA production due to the lag time between when the sample is taken and when the final results can be tabulated. Typically, the samples are taken and transported to the analytical laboratory. Samples are then usually batched (since processing is such a labor-intensive process) for processing. They are extracted either organically or using super critical fluid extraction. Extracted samples are then converted to their methyl esters by a chemical process and finally extracted samples are injected sequentially to be analyzed chromatographically. The entire analytical process will typically require at least a full work shift to complete, and consequently data used to determine conditions in a fermentation tank will be at least 6-8 hours old (often much older due to the batch running of samples). This causes some problems when changes in metabolic activities take place in a much more rapid time frame. In contrast, the rapid detection made possible by the methods of the present invention would allow producers to follow fermentations for DHA on a real time or near real time basis such that the fermentor could be harvested at its actual peak of DHA production versus an historical peak (obtained from prior fermentations) or a "time-shifted" peak where one uses data 6-8 hours old to determine what is currently happening in the fermentor.

In view of the association of DHA with numerous and diverse neurological disorders, as well as the positive effects of DHA treatment on those disorders and the association of increased maternal and infant DHA levels with improved infant neural development, it is desirable to detect and quantify DHA levels in biological samples. Similarly to detection and quantification of DHA in production samples, detection and quantification of DHA in biological samples via presently available methods is time consuming and complicated (*See, e.g.*, Nelson, et al., 1997, *Lipids*, **32:**1137-1146).

Assays adapted for physicians' office laboratories (POLs) and point of care diagnostics (POC) provide for rapid feedback to the consulting physician and, with the increasing recognition of the importance of DHA status, could be a routine analysis performed to assess the DHA levels in a patient with a specific disease (e.g., retinitis pigmentosa) or metabolic state (e.g., pregnancy).

Other objects and advantages of the invention are set forth in the description which follows, and additional objects and advantages will be apparent to a skilled worker in the art from this description. or may be learned from the practice of the invention.

DETAILED DESCRIPTION OF THE INVENTION

As embodied and broadly described herein, the present invention depends on the use of a protein which binds to DHA with high specificity in order to detect or quantify DHA in a sample containing DHA and other fatty acids.

General Methods

The practice of the present invention employs, unless otherwise indicated, conventional biochemistry, clinical chemistry, immunology, molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are well known to the skilled worker and are explained fully in the literature. (*See, e.g.*, Nakamura, et al., 1992, "Immunochemical Assays and Biosensor Technology for the 1990s," *American Society for Microbiology*, Washington, D.C.; Maniatis, et al., *Molecular Cloning: A Laboratory Manual*, 1982; *DNA Cloning: A Practical Approach*, Volumes I and II (D.N. Glover, ed., 1985); *Oligonucleotide Synthesis*, (M.J. Gait, ed., 1984); *Nucleic Acid Hybridization*, (B.D. Hames & S.J. Higgins, eds., 1985); *Transcription and Translation*, (B.D. Hames & S.J. Higgins, eds., 1984); *Animal Cell Culture*, (R.I. Freshney, ed., 1986); *Immobilized Cells and Enzymes*, (IRL Press, 1986); B. Perbal, *A Practical Guide to Molecular Cloning*, (1984), and Sambrook, et al., *Molecular Cloning: A Laboratory Manual*, (1989).

Obtaining a Sample

The method of this invention can be used to evaluate samples from individuals, samples from production systems, or any other sample source, the DHA content of which is desired to be determined. These samples include, but are not limited to biological samples; samples of human tissue; samples of foods; and samples of microbial or algal lysates or other DHA production systems.

As used herein, a "biological sample" refers to a sample of tissue or fluid isolated from a individual, including but not limited to, for example, plasma, serum, spinal fluid, lymph fluid, the external sections of the skin, respiratory, intestinal, and

genitourinary tracts, tears, nipple aspirate fluid, saliva, milk, blood cells, tumors, organs, and also samples of *in vivo* cell culture constituents (including but not limited to conditioned medium resulting from the growth of cells in cell culture medium, putatively virally infected cells, recombinant cells, and cell components). Biological samples may be obtained by expression of fluid, phlebotomy, punch biopsy, etc. The listed sampling methods are a non-exhaustive list provided by way of example, and other sampling methods will be apparent to the skilled clinician.

"Human tissue" is an aggregate of human cells which may constitute a solid mass. This term also encompasses a suspension of human cells, such as blood cells or a human cell line.

Aqueous solutions or suspensions containing DHA may typically be assayed directly in the aqueous solution. If the DHA-containing material is within cells, the cells can be broken using any suitable method, such as freeze fracture, sonication, cavitation (French pressure cell or Microfluidics), glass bead pulverization, homogenization, or other methods in the literature. Detergent solubilization may also be beneficial. The broken cells may be removed from the sample by centrifugation and the supernatant analyzed or, alternatively, a crude cell lysate may be analyzed without centrifugation, relying on the assay to remove unwanted background associated with cell debris. In view of the aqueous/lipid phase separation that tends to occur in samples containing significant amounts of lipid, the samples should be well mixed. However, the free fatty acids in the solution or in micelles can typically be accessed by the DHA-binding protein in this format.

Complex lipid materials containing DHA moieties which bind the DHA-binding protein may be assayed directly. Alternatively, complex lipid materials may be saponified to release DHA as a free fatty acid to facilitate the assay. Methods of saponification are within the skill of the ordinary worker in the art. As an example, saponification may be accomplished by treating the sample with base (e.g., 8 M KOH in methanol) to release DHA as a free fatty acid. After adjustment of the pH, the DHA can be assayed directly.

Alternatively, the sample can be extracted with an organic solvent. In this alternative, the DHA would be found in the organic fraction. Where the solvent used does not interfere with binding between the DHA and the DHA-binding

protein, the assay may be carried out in the organic fraction. Where the solvent used does interfere with binding between DHA and the DHA-binding protein, the DHA can be re-extracted into an aqueous fraction, then assayed in the aqueous fraction.

In yet another embodiment, the DHA-containing sample is treated with PUFA-specific lipases which release DHA as a free fatty acid. Such treatment is done in an aqueous environment, then the assay is carried out directly in the aqueous environment. Detergents that do not disrupt the BLBP but do more easily mobilize the fatty acid or lipid could be used to homogenize the sample to provide more complete presentation of the fatty acid to its specific binding partner FABP.

DHA Binding Protein

DHA in the sample can be bound by any protein which will bind DHA selectively in the presence of other fatty acids. Hereinafter, the protein to bind DHA will be referred to as the "DHA-binding protein." Preferably, the DHA-binding protein has an affinity for DHA which is at least one half of one order of magnitude greater than the affinity of the protein for other fatty acids. More preferably, the DHA-binding protein has an affinity for DHA which is at least a factor of 5 greater than its affinity for other fatty acids. Even more preferably, the DHA-binding protein has an affinity for DHA which is at least a factor of 10 times as great as its affinity for other fatty acids.

An example of a suitable protein to bind DHA is BLBP, which binds DHA with a dissociation constant (Kd) of approximately 10 nM, which represents an affinity nearly as strong as that of antibodies for their antigens. This affinity is more than one order of magnitude higher than the affinity of BLBP for oleic acid or arachidonic acid. Furthermore, competition assays demonstrated that DHA is a more effective competitor with oleic acid for binding to BLBP than are other fatty acids, such as arachidonic and 11-*cis*-eicosenoic acid (Xu, 1996).

BLBP may be obtained from animals, preferably vertebrates, and more preferably mammals. Examples of typical sources include sheep, goat, pig, cow, rabbit, rat, mouse, pigeon, etc. BLBP for use in this invention can be from any species producing BLBP that meets the above-mentioned criteria for a DHA-binding protein.

BLBP, also known as B-FABP, is encoded by a gene designated *Fabpb*, or GC9 (GenBank U04827) (Kurtz, 1994). Procedures for expression of proteins of defined sequence are well known in the art. A DNA sequence encoding a protein can be synthesized chemically or prepared from the wild-type sequence by one of several approaches, including primer extension, linker insertion and PCR (see, e.g., Sambrook, et al.). Protein may be prepared by placing the coding sequence for the polypeptide in a vector under the control of a promoter, so that the DNA sequence is transcribed into RNA and translated into protein in a host cell transformed by this (expression) vector. The protein may be produced recombinantly by growing host cells transfected by an expression vector containing the coding sequence for the protein under conditions whereby the polypeptide is expressed. The selection of the appropriate growth conditions is within the skill of the art. BLBP can be produced using standard methods of expression of recombinant proteins. For example, the cDNA can be cloned into a plasmid vector, expressed in cells, and isolated (Xu, 1996). Recombinantly produced BLBP, as contemplated herein, includes BLBP sequences which are altered by standard mutagenesis, including alterations in recombinant DNA sequence, or BLBP sequences fused to other polypeptide sequences to form a fusion protein, so long as the BLBP meets the above criteria for a DHA-Binding Protein.

Antibodies against DHA may be produced via any method used for producing antibodies. Antibodies which are specifically reactive with the fatty acid or fatty acid-protein complex of this invention may be obtained in a number of ways which will be readily apparent to those skilled in the art (*see. e.g.*, Sambrook et al.). In one alternative, the fatty acid-protein complex may be injected into an animal as an immunogen to elicit polyclonal antibody production. The fatty acid-protein complex obtained above may be used directly or after chemical stabilization of the complex. Purification of the antibodies can be accomplished by selective binding from the serum, for instance by binding the antibody to a BLBP-GST fusion protein bound to a column (Feng, 1994).

In another alternative, monoclonal antibodies specifically immunoreactive with the protein may be prepared according to well known methods (*See, e.g.*, Kohler, et al., 1976, *Eur. J. Immunol.*, **6**:611), using the DHA-Binding Protein, fatty

acid or fatty acid-protein complex of this invention as an immunogen, using it for selection or using it for both functions. These and other methods for preparing antibodies that are specifically immunoreactive with the recombinant protein of this invention are easily within the skill of the ordinary worker in the art.

Detection of the DHA-Binding Protein - DHA Complex

After binding the DHA-binding protein to the DHA, the resulting complex is detected using any detection system which will detect and/or quantify by differentiating between bound and unbound DHA. Such detection systems are within the skill of the ordinary worker in the art. The detection system can be quantitative or qualitative. The detection can occur in a heterogeneous or homogeneous format and in liquid or solid phase. The complex may be detected directly or indirectly. In a preferred mode, detection involves direct detection of a complex containing DHA.

Any assay format known in the art may be utilized, including without limitation, homogeneous assays, heterogeneous assays, competitive assays, and sandwich assays. In homogeneous assays, binding of the two binding partners (*e.g.*, ligand and receptor) influences activity of the label; no separation of bound and unbound reagents is required. In heterogeneous assays, separation of bound and free reagents is required to determine the amount of binding which has occurred. Quantification of such assays can be accomplished by any appropriate quantification means, including but not limited to fluorescent labeling, radiolabeling, chemiluminescence, or photometric, fluorometric or optoelectronic means.

The art is replete with procedures for quantifying the binding between a high molecular weight entity (*e.g.*, a protein) and a low molecular weight entity (*i.e.*, a small molecule, such as a fatty acid). Typically, such procedures make use of the size difference between the small molecule and the complex formed when the small molecule is bound to a high molecular weight molecule.

For example, in one alternative, the high molecular weight complex may be separated from the unbound small molecule by ultrafiltration or molecular sieve chromatography. Alternatively, in another embodiment, an analog of the small molecule which emits a signal sensitive to molecular motion may be bound, and changing signal monitored to detect the change in molecular motion as a result of

binding. In a preferred mode of the invention, a fluorescent analog of DHA competes with DHA in a sample for binding to BLBP. The fluorescent analog causes less depolarization when bound in a large molecular weight complex than when freely tumbling in solution, and so binding can be detected by fluorescence polarization, and reduction in the bound signal is correlated to displacement of the analog by DHA from the sample. Alternatively, a fluorescent analog of a fatty acid with lower affinity for BLBP could be utilized as the fluorescent probe.

Particularly useful assays employ a solid phase, to which either the analyte or its specific binding partner is attached. The solid phase facilitates the separation of bound from free phases in a heterogeneous specific binding assay. Popular solid phase reactions include Southern, northern, and western blotting assays. Any suitable solid phase can be used, chosen for its acceptability in a particular assay environment. These include synthetic membranes, polymers, microparticles, and glass.

The DHA-binding protein may be immobilized to a manufactured solid support, such as a microtitration dish, microparticle, polymeric bead, polymer matrix, polymer, synthetic membrane, liposome, glass, etc. The DHA-binding protein may be modified, conjugated, or stabilized, prior to attachment. The attachment may be covalent or noncovalent, specific or non-specific. The method of attachment may be optimized to achieve a preferred orientation of the protein relative to the solid surface. For some applications, it may be desirable that the protein or lipid binding partner be attached in an ordered array, such as in a grid or other pattern.

Complex Specific Binding Partners

A nucleic acid aptamer or protein may be constructed which binds the DHA-binding protein-DHA complex with high specificity. After binding between DHA and DHA binding protein to form a complex, the bound material will typically be purified away from the free DHA to get a purified fraction. It may be helpful to gently cross-link this fraction to maintain the DHA/BLBP complex. The purified material can be used as the antigenic material for production of antibodies in any antibody generation model (animal, cell culture or bacterial). Antibodies that recognize only the bound complex may be culled from the antibodies produced by

affinity purification methods. For example, unbound BLBP may be used to remove all of the antibody specific for BLBP. Then DHA/BLBP complex can be used to concentrate those antibodies with affinity to the complex. Repetitive affinity purification will provide a strongly selective antibody for bound complex only. The results of this would be a DHA/BLBP complex specific antibody (hereinafter "DBIgG").

A series of panning procedures (modeled on phage panning) on microplates would achieve the same thing. Here DHA/BLBP would be bound to a plate. A reaction mix would be added that contained BLBP (without bound DHA) to compete for non-specific antibodies. The antibodies developed as described above would be added and only those that specifically recognize the BLBP/DHA would be concentrated on repeated passes over this procedure.

A synthetic nucleic acid aptamer to the DHA/Brain Lipid Binding Protein complex (hereinafter "DBAptamer") can be made in a combinatorial fashion. This will then be analyzed to assure specificity. It will in effect be a nucleic acid based pseudo-antibody for pseudo-immunological binding assays (Kawazoe, et al., 1997, *Biotechnol. Prog.* **13**:873-874; O'Rourke, 1997, *Clinical Laboratory News*, **1**:10-11; Griffin, et al., U.S. Patent 5,756,291; Famulok, et al., 1993, *Nucleic Acids and Molecular Biology*, 271-284, F. Eckstein, D.M.J. Lilley (eds.); Cubicciotti, U.S. Patent 5,756,296; Cubicciotti, U.S. Patent 5,739,305; Burke, et al., U.S. Patent 5,637,459).

In one embodiment, DHA is detected in a sample using the DBIgG or the DBAptamer. A microplate is activated with glutaraldehyde and then BLBP is added to the system and allowed to covalently bind to the bottom of the well. Alternatively, the BLBP can be put on the plate by passive adsorption or by a BLBP specific capture antibody. To facilitate binding to the plate, variant structures of BLBP may be produced recombinantly. For example, binding may be improved via introduction of a cysteine which may be coupled to a SH which is bound to a support. Procedures for preparing such recombinant proteins are within the skill of one in the art, particularly in view of the guidance provided above. After an appropriate incubation of the BLBP (from 30 min to overnight), the wells are washed with buffer and at this point are either stored or used for a sandwich assay.

This form of heterogeneous assay would be done by adding sample to the system in a form that allows easy access of the DHA to the BLBP. Optionally, the addition of detergents or solvent may be used to get the material in a form that is more accessible for binding. After binding, the unbound DHA will be a washed away along with debris and fatty acids that are not tightly bound to the BLBP. Optionally, reagents may be added to stabilize the binding before washing. Suitable reagents include, but are not limited to QPREP (Beckman Coulter Corporation), paraformaldehyde, formaldehyde, FACSLYSE (Becton Dickinson Corporation), glutaraldehyde and other fixatives.

The washed plates, with a complex containing the DBIgG or DBAptamer bound, are then reacted to a signal generating system (*e.g.*, enzyme, fluor, nanogold, radioisotope, etc.) which binds only to the DHA bound by the BLBP and accumulates in proportion to the DHA added. This is incubated for an appropriate length of time and then washed to remove unbound label. The plates are read by the appropriate device. The concentration of DHA in the sample will be proportional to the signal increase observed.

In a preferred embodiment, the DHA-Binding Protein-DHA complex may also be detected by direct labeling methods. For example, samples are labeled either with a general stain, such as Nile red, for fatty acids or some form of label that is more specific for DHA or the LC-PUFAs. The samples are then added to wells and labeled DHA is allowed to bind to BLBP bound (as above) to the microplates. The plates are washed and read directly in the appropriate instrument. Alternatively, the samples may be added to the wells and the unbound fatty acids washed away, followed by the addition of the stain to label bound DHA. Suitable labels include, but are not limited to, fluors like Nile red that non-specifically bind to lipids, in which case all the lipids would be labeled, but only the labeled DHA would be retained by the BLBP and detected when placed in a plate reader.

The DHA-Binding Protein DHA complex may also be detected by displacement assay. For example, DHA labeled with a labeling compound (fluor, enzyme, radiolabel, stable isotopic label) is allowed to bind to BLBP that has been bound covalently or non-covalently to the plate. This is then washed extensively and read to determine a baseline value related to bound, labeled material. Samples

containing DHA are added to the system as well as appropriate negative controls. As the labeled DHA is exchanged with the unlabeled DHA in the samples, an overall decrease in the labeling seen in each well will be observed. This will be proportional to the DHA in the sample. Alternatively, another unlabeled fatty acid with lower affinity for BLBP than DHA may be used in displacement assays.

The assays of this invention may be used to monitor progression of disease states in which DHA plays a role, or to monitor levels of DHA in tissue, fluids and other biological samples over time. These assays may also be used to monitor production processes, such as microbial fermentations described in U.S. Patent No. 5,407,957, with the advantage that the assay allows for more rapid analysis which is useful for accurate harvesting in production and for rapid diagnostic testing. These assays may also be used for determination of DHA content in foods.

Also within the contemplation of this invention is a self-contained kit for detection of DHA in either a regulated or non-regulated application. The kit could be in a number of different formats, which are within the reach of a routine worker in the art, particularly by analogy to immunoassay kits. Examples of such formats include, but are not limited to dipstick, glass slide, array, silicon wafer, patterned chip, and microplate formats. The microplates are particularly useful for research or industrial applications. The dipsticks are particularly useful for industrial plants or in-office type applications. Some high density formats can be used in drug discovery or diagnostic applications. Kits may also contain apparatus and/or reagents for pretreatment of the samples.

Fusion Proteins

Proteins such a BLBP (brain lipid binding protein) and retinoid binding protein have extremely high affinities for DHA (10^{-8}M) that rival antibody affinities. The gene for these fatty acid binding proteins might be expressed recombinantly in other organisms while retaining their binding properties. These characteristics could make FABPs amendable to be used as a gene reporting system and as a tool for the affinity purification of fusion proteins. Examples of other similar systems are Streptag and His-Tag purification. A possible advantage of a DHA binding fusion protein is that a hydrophobic ligand may play in the purification of hydrophobic membrane bound proteins. Another advantage is the use of labeled DHA to quantiate the amount of gene expression in a given system. This is amenable for high throughput screening (HTS) and bioinformatic applications. In addition, the hydrophobicity of DHA may lend itself to labeling with infrared dyes (normally very hydrophobic) to assess the success or failure of genetic therapies inside animal and eventually human models.

Therefore, this invention also encompases the use of the gene sequence for the BLBP or other FABP as an expression cassette as well as an affinity purification system. DNA encoding one or more other polypeptides can be fused in frame with the FABP sequence for expression of a fusion protein capable of binding fatty acids as well as other activities. The insertion/transformation of genetic material into cells (bacterial, algal, fungal, mammalian) for the express purpose of creating gene fusion proteins and gene reporting systems that specifically bind DHA and other fatty acids could provide a generally applicable technique. (1) In particular it could prove of importance for therapeutic applications. For example, one may express a proteinaceous enzyme that functions in the brain or retina and be able to target its action more precisely using the fusion protein bound to DHA. Alternatively, one could add the fusion protein and allow it to bind *in situ* with the polyunsaturated fatty acid which could then serve as a targeting moiety for the therapeutic. It will be necessary to make sure that the fatty acid bound to the binding protein is specific for particular organs, but any number of fatty acid binding proteins could be used. (2) This invention could also be of importance for situations where a hydrophobic protein is being expressed, association of this to a lipid binding protein could

provide a lipid capture system to keep the hydrophobic fusion protein in solution. (3) The fusion protein system comprising FABP could be used for purification of the fusion protein by binding to a hydrophobic (DHA or some other ligand that recognizes the FABP with high affinity) surface.

This system provides advantages since it (FABP) is made for a hydrophobic ligand and no other expression system at this time is so designed. Use of this system will facilitate detection of gene expression and affinity purification of FABP fusion proteins that contain this functional group that binds polyunsaturated fatty acids. It will provide interesting therapeutic applications for specific targets pinpointed with specific binding proteins fused on them. This system may be used for isolating membrane bound proteins and hydrophobic proteins that are not easily affinity purified when used as an expression system. Due to the apparent localization in the retina and brain of BLBP and the localized concentration of FABPs, this system could be used to isolate and target fusion proteins of therapeutic importance to specific target membranes using either a fatty acid complex, or it could be used by just adding the fusion protein and allowing it to ligate to endogenous fatty acids. Thus, this system provides specificity and a delivery mechanism in the blood for pharmaceuticals.

EXAMPLES

In order to facilitate a more complete understanding of the invention, a number of Examples are provided below. However, the scope of the invention is not limited to specific embodiments disclosed in these Examples, which are for purposes of illustration only.

Example 1 -- Assay using BLBP and aqueous processing for analysis of DHA

An aqueous sample containing DHA is treated with 8 M KOH to degrade the complex lipids into free fatty acids. This material is stirred for 5 h at room temperature. This is then adjusted to pH 6.5 with HCl and stored until use at -20°C.

The assay is a displacement assay done in the following manner. A microtitration plate is pre-coated with BLBP at 10-50 μg/ml in 10 mM sodium phosphate buffer (pH 7.0) for between 4 and 24 h at 4°C. The BLBP on the plate is washed exhaustively with PBS. A fluorescent analog of DHA is pre-bound to fill the sites available on the immobilized BLBP. The BLBP with bound fluorescent

analog is washed, then a sample (the DHA content of which is desired to be analyzed) is added to the plate. Free DHA from the sample displaces the bound DHA, if the concentration is higher than the affinity binding constant. Free, fluorescently-labeled DHA is washed off, and a decrease in bound fluorescence can be directly correlated to the DHA in the sample (displacement assay).

Example 2 -- Organic extract

A DHA-Binding Protein which is stable in an organic solvent is immobilized on a surface such as, for example, a chamber or microtitration dish. A DHA-Binding Protein which is stable in the organic solvent in its immobilized state, but not in its free state, may also be used. DHA in a sample is extracted into the organic solvent, and the extract is added to the chamber or microtitration well and allowed to bind to the DHA-Binding Protein. After reacting and binding, the bound material is washed with organic solvent, then optionally with an aqueous buffer solution such as phosphate buffered saline (PBS).

Antibody that recognizes the DHA-Binding Protein-DHA complex will be allowed to react with the bound material, such that any complex containing DHA bound to DHA-Binding Protein will be marked with a specific antibody. This antibody may be visualized, for example, by prior biotinylation of the antibody, then detection with an enzyme-linked system (*e.g.*, alkaline phosphatase) where optical absorbance, fluorescence or luminescence can be generated on the basis of enzyme activity. The materials generated are measured using the appropriate plate reader, and the amount of DHA present will correspond to the readings taken. The procedure may be carried out with various organic solvents, including, but not limited to IPA, hexane, etc., using a DHA-Binding Protein that is stable in that solvent.

Example 3 -- Magnetic bead based binding assay

In this solid phase an assay, DHA-Binding Protein is immobilized to paramagnetic beads using a standard cross-linking chemistry for proteins, such as the glutaraldehyde one-step system. The magnetic beads are made to have exposed amine groups, then DHA-Binding Protein is immobilized onto them. A small amount of cross-linker is added to activate the amines and link one amine to another, forming a bridge between the magnetic bead and the DHA-Binding Protein. This is

usually done with an excess of one of the components to drive the desired reaction and avoid an undesired reaction. A combination of 3-5 fold more DHA-Binding Protein than magnetic beads is an example of a suitable ratio. The beads and protein are reacted, then quenched with an amine- containing material, such as lysine, glycine or glycylglycine. The beads are purified by repeated washing in clean buffer using a magnet to facilitate separation.

Once the beads are purified, analogs of DHA or other fatty acids with lesser affinity are added to the vial to pre-label the beads. Examples of suitable labels include DHA labeled with fluorescent, radioactive, stable isotopic, colorimetric or any other suitable label. If ^{14}C radioisotopically labeled DHA is used, ^{14}C-DHA is bound to the beads and washed repeatedly to remove non-specifically bound label. It may be helpful to add oleic acid to the washes in order to remove non-specifically bound material.

Aliquots of the labeled beads are put in a glass tube and used for the assay. Enough label is used that a substantial radioactive signal is produced. In a quantitative assay, a substantial radioactive signal is one which allows degree of displacement to be quantified. In a qualitative assay, a substantial radioactive signal is one which allows displacement to be observed. As sample material is added to this reagent system, non-labeled DHA from the sample DHA competes with ^{14}C-DHA on the DHA-Binding Protein. Displacement occurs in proportion to the concentration of DHA in the sample. The tube will be cleared by attracting the beads to a magnet, and the cleared solution evaluated for an increase in radioactivity. Any ^{14}C-DHA released from the loaded beads will correspond to free DHA in the sample that displaced the label (over background, which is measured before adding the sample to the beads).

Example 4 -- Homogeneous assay based on fluorescence polarization

Samples prepared as described in Example 1 are mixed with a solution containing DHA-Binding Protein bound to fluorescently labeled DHA. Fluorescence polarization spectroscopy is used to follow the increase in depolarized fluorescence resulting from DHA displacement of labeled DHA from its binding site on the DHA-Binding Protein. As the label is released, the fatty acid begins tumbling more rapidly and results in more of the polarized light becoming depolarized. The

slowly tumbling DHA-Binding Protein-DHA complex does not depolarize light to the same degree. This is a displacement assay that requires conditions that mix the samples well (to maintain lipids well mixed with the aqueous solution) in order to produce a significant change in polarization caused by the release of the small, fluorescently labeled fatty acid. In a typical assay configuration conditions are selected to minimize or obviate formation of micelles by the free fatty acids. Alternatively, detergent or lipid micelles may be added to take up the released fatty acid and/or label, thereby decreasing depolarization.

Example 5 -- Latex bead-based assay for blood and plasma DHA levels

DHA-Binding Protein is bound to latex beads. This binding may be accomplished via covalent binding or non-covalent binding methods.

Red cells in a blood sample are lysed. The fatty acids may be processed by such methods as saponification or lipase treatment, or the sample may be assayed directly.

The fatty acids from the sample are allowed to react with the DHA-Binding Protein on the latex beads. The beads are washed in PBS, then allowed to react with antibody specific for the DHA-Binding Protein-DHA complex; the antibodies are labeled prior to the reaction, either directly with a marker (such as a fluorescent dye, radioisotope, colorimetric, or other suitable label) or with another easily detectable marker, such as fluorescein, digoxin, biotin, etc. The beads are washed with PBS buffer. If the marker used to label the antibodies so requires, the beads are allowed to react with an appropriate secondary reagent (*e.g.*, streptavidin labeled dye, anti-fluorescein IgG, anti-digoxin IgG, etc.), then are washed again.

The label on the beads is read on an appropriate instrument. If the reading is done, for example, on a flow cytometer, a fluorescent dye as a primary or secondary label may be used, and the proper channel is used to image a specific bead. As DHA enters into the sample, the beads will either be brighter, or more of the beads will be labeled as a percentage of the total number of beads present. The amount of DHA in the red blood cell membrane may be compared to plasma levels by omitting the cell lysis and saponification steps, to investigate how differing levels correlate to the overall DHA status of the patient. This assay can also be carried out with isolated RBC membranes and/ or with isolated plasma phospholipids.

Example 6 -- Hydrophobic bead capture of analyte fatty acid

A sample is treated with hydrophobic beads to capture all hydrophobic compounds on the bead surface. The beads are then washed repeatedly in aqueous buffer such as PBS to remove all aqueously soluble materials. Hydrophobic residues should be tightly adsorbed to the bead. At this point, fluorescently labeled FABP is added to the samples (alternative labels may be used, with appropriate alternative imaging instruments) and reacted to allow the desired FABP to be bound to its appropriate fatty acid. The beads are then washed repeatedly in aqueous buffer to remove excess label and read in either a microplate reader or flow cytometer. Increases in label on the bead over a control bead (where an unreactive fatty acid was applied to the hydrophobic bead as negative control or large excess of the free fatty acid that would bind to the FABP were it in the sample as positive control) would indicate the presence and amount of the fatty acid in the sample.

Example 7 -- Microplate immobilization of analyte fatty acid for detection of BLBP or other FABP in sample

Add DHA or fatty acid of choice in the presence of N,N'-dicyclohexylcarbodiimide to react with free hydroxyls on the plate well surface. The plate can be washed with solvent and/or aqueous buffers to ready it for analysis and to remove non-covalently bound material. Samples are added in aqueous buffer to the wells, and the specific binding protein present in the sample will bind to the covalently bound fatty acid. This can then be visualized by addition of antibody to either BLBP or the BLCP-DHA complex. This antibody is then detected by an antibody to the species which produced the BLBP antibody (for example mouse) labeled with a signal generating species (for example HRP). This is then washed exhaustively and substrate added to react with HRP to develop a color that can be detected on a visible microplate reader.

Example 8 -- Detection for DHA using immobilized ligand

The fatty acid is bound as above and a reaction mixture containing fluorescently-labeled BLBP is utilized. Sample is mixed with standard solutions containing the label-BLBP conjugate. The amount of either fatty acid or free BLBP present in the sample will be reflected in a decrease of the observed fluorescence in

the well. By comparison, with proper standards, this can provide quantitative determination of the BLBP or fatty acid being observed.

Example 9 -- Homogeneous detection of DHA

BLBP labeled with cross-linked-Allophycocyanin (APC) and antibody to the BLBP/DHA complex that had been labeled with europium cryptate are added to a plate in aqueous buffer. Samples or standards are added to this in a defined volume of aqueous buffer. In the absence of free DHA, no binding of the cross-linked-APC will occur, and therefore no energy transfer will be observed from Eu^{3+} to cross-linked-APC (as measured by an increase in fluorescence emission at 660 nm on excitation of the Eu^{3+}. This is done in a time-resolved mode, using instruments such as the Wallac Victor II or Packard Discovery plate readers. When DHA is present in the sample it will be bound by the BLBP forming the BLBP/DHA complex. This complex will then be recognized by the antibody to the complex and will then bring the Eu^{3+} cryptate to a distance permissive to resonance energy transfer between these fluors. An increase in the emission at 660 nm will correlate with an increase in the presence of DHA in the sample or standard.

Example 10 -- An assay system that distinguishes between free BLBP and a DHA/BLBP complex.

This assay could be used to determine the metabolic state of a patient. The assay uses various antibodies that recognize (1) free BLBP, (2) DHA-bound BLBP, and (3) BLBP in general (whether or not DHA is bound). The assay may be done as a sandwich assay.

A general recognition antibody that would be used to capture any BLBP (with or without bound DHA), would be used to coat a plate in 10 mM sodium carbonate (pH 9.0) buffer for 3h at room temperature. This would then be washed with PBS. The plate would be blocked with a blocking buffer containing 10 mM sodium phosphate (pH 7.2), 1% bovine serum albumin, 0.5% casein and 0.05% sodium azide for 2h at 37°C; the plate would then be washed with PBS three times.

The general recognition antibody would be used to pull down any BLBP in the standard or sample added to the well in PBS-based buffer. This would be allowed to bind for 30 minutes at 37°C. The plate would then be washed extensively with PBS to remove non-specifically bound material.

Two conjugates labeled with different fluorescent probes would be used for 2-color assays. Two appropriate fluors would be fluorescein and R-PE; in this example, an anti-BLBP:fluorescein conjugate and an anti-BLBP/DHA:R-PE conjugate. These are added as a cocktail in assay buffer (100 mM sodium phosphate (pH 7.2), 150 mM NaCl, 0.1% BSA, 0.05% sodium azide) at about 1 µg/mL of protein and allowed to react with the bound BLBP or BLBP/DHA complex. The reaction would be done at 37°C for 30 min with shaking. The plate would be washed three times with PBS and then read on a microplate reader at the appropriate emission wavelengths (519 and 573 nm for fluorescein and R-PE, respectively). The data would provide a quantitative estimate of the BLBP present and whether it was bound to ligand or not. This could have importance as DHA is further correlated to human development and metabolism.

For purposes of clarity of understanding, the foregoing invention has been described in some detail by way of illustration and example in conjunction with specific embodiments, although other aspects, advantages and modifications will be

apparent to those skilled in the art to which the invention pertains. The foregoing description and examples are intended to illustrate, but not limit the scope of the invention. Modifications of the above-described modes for carrying out the invention that are apparent to persons of skill in the fields of analytical biochemistry, molecular biology, immunology, and/or related fields are intended to be within the scope of the invention, which is limited only by the appended claims.

All publications and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

CLAIMS:

1. A method for detecting the presence or amount of docosahexaenoic acid (DHA) in a sample, optionally in the presence of other fatty acids, said method comprising

contacting a sample with a protein having differential binding specificity for DHA over other fatty acids under conditions where DHA will bind to the protein to form a DHA-protein complex; and

detecting binding between the protein and DHA from the sample.

2. The method of claim 1, wherein said step of detecting comprises detecting the DHA-protein complex.

3. The method of claim 2, wherein the DHA-protein complex is detected through binding by a protein or DNA aptamer specific for the complex.

4. The method of claim 1, wherein binding between the protein and DHA is detected by measuring bound and/or unbound DHA.

5. The method of claim 1, wherein said step of contacting is carried out in the presence of a labeled analog of DHA.

6. The method of claim 1, wherein the protein has an affinity for DHA that is at least half an order of magnitude greater than its affinity for other fatty acids.

7. The method of claim 1, wherein the protein is Brain Lipid Binding Protein (BLBP).

8. The method of claim 7, wherein the protein is BLBP produced recombinantly.

9. The method of claim 1, wherein the protein is immobilized.

10. The method of claim 1, wherein the sample comprises biological material.

11. The method of claim 10, wherein the biological material is selected from microorganisms, fractions of cells, fish tissue, mammalian tissue, and biological fluids.

12. A method of claim 1, further comprising a step of hydrolyzing complex lipids to release DHA residues as free DHA.

13. The method of claim 12, wherein said hydrolyzing is non-enzymatic.

14. A kit for detection of DHA in a sample comprising:

a protein having differential binding specificity for DHA over other fatty acids; and

means for detecting formation of a complex between said protein and DHA.

15. The kit of claim 14, wherein said protein is BLBP.

16. The kit of claim 15, wherein said protein is produced recombinantly.

17. The kit of claim 14, further comprising reagent means for saponifying complex lipids.

18. The kit of claim 14, wherein the protein is immobilized.

19. A recombinant fusion protein comprising at least a portion of the sequence of a fatty acid binding protein, wherein said recombinant protein specifically binds fatty acid.

20. The recombinant fusion protein of claim 19, wherein the fatty acid binding protein is BLBP.

DECLARATION AND POWER OF ATTORNEY FOR PATENT APPLICATION

As the below named inventor, I hereby declare that:

My residence, post office address and citizenship is as stated below next to my name;

I believe that I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

SPECIFIC BINDING ASSAY FOR DOCOSAHEXAENOIC ACID

the specification of which: ☐ is attached hereto.
☒ was filed on: **September 26, 2001**
as Application No.: **09/937,477**
and was amended on: _____ (if applicable).

I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above. I acknowledge the duty to disclose information which is material to patentability as defined in 37 C.F.R. § 1.56.

And I hereby authorize and request our agents, Brobeck, Phleger & Harrison LLP, whose address is set forth below, to insert above, the filing date and application number of said application when known.

Prior Foreign Application(s)

I hereby claim foreign priority benefits under Title 35, United States Code, § 119(a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate, or § 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below any foreign application(s) for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Country	Application Number	Date of Filing (day, month, year)	Date of Issue (day, month, year)	Priority Claimed	
				Yes ☐	No ☐
				Yes ☐	No ☐

Prior Provisional Application(s)

I hereby claim the benefit under Title 35, United States Code § 119(e) of any United States provisional application(s) listed below:

Application Number	Date of Filing (day, month, year)
60/126,513	March 26, 1999

Prior United States Application(s)

I hereby claim the benefit under Title 35, United States Code, § 120 of any United States application(s), or § 365(c) of any PCT international application designating the United States of America, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, § 1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

Application Number	Date of Filing (day, month, year)	Status - Patented, Pending, Abandoned
PCT/US00/07989	March 27, 2000	Pending

And I hereby appoint, both jointly and severally, as my attorneys with full power of substitution and revocation, to prosecute this application and to transact all business in the Patent and Trademark Office connected herewith the following attorneys, their registration numbers being listed after their names:

Rodger L. Tate, Registration No. 27,399; Anthony W. Shaw, Registration No. 30,104; Cono A. Carrano, Registration No. 39,623; Laurence H. Posorske, Registration No. 34,698; Robert A. King, Registration No. 42,738; Craig L. Puckett, Reg. No. 43,023; and Trevor Q. Coddington, Registration No. 46,633.

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine and imprisonment, or both, under 18 U.S.C. § 1001, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Signature _____ Date 9 Jan 2002

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